Susceptibility of the Mammary Gland to Carcinogenesis

II. Pregnancy Interruption as a Risk Factor in Tumor Incidence

Jose Russo, MD, and Irma H. Russo, MD

In the rat, pregnancy and lactation prior to carcinogen administration protect the mammary gland from developing carcinomas and benign lesions. In this study, the influence of pregnancy interruption versus full pregnancy and pregnancy plus lactation on the incidence of carcinomas and benign lesions was studied in the mammary glands of rats treated with 7,12-dimethylbenz(a)anthracene (DMBA). Fifty-nine Sprague-Dawley rats were separated into 5 groups: I) rats that had had one pregnancy and one lactation; II) rats that had had one pregnancy without lactation; III) rats that had had pregnancy interrupted at the 12th day of gestation; IV) age-matched virgin rats as a control for Group I; and V) age-matched virgin rats as a control for groups II and III. The 5 groups received a single intragastric dose of DMBA (10 mg/100 g body weight), with the exception of 2 animals per group, which were killed 1 hour after an intraperitoneal injection of 2.5 μ Ci ^{3}H-thymidine/g body weight. The number of labeled nuclei per 100 cells (DNA labeling index, LI) was counted in terminal end buds (TEBs), terminal ducts (TDs), and alveolar buds (ABs) of the glands. The number of structures and the DNA-LI were correlated with the incidence of tumors at 22 weeks after DMBA. Pregnancy, with or without lactation, resulted in elimination of TEBs and reduction in the DNA-LI of TDs and ABs. These groups did not develop carcinomas. After the interruption of pregnancy the mammary gland contained numerous TEBs, with a high DNA-LI; 77% of these animals developed carcinomas, and all of them developed benign lesions. Therefore, while pregnancy and lactation protected the mammary gland from developing carcinomas and benign lesions by induction of full differentiation, pregnancy interruption did not elicit sufficient differentiation in the gland to be protective, and these animals were at the same risk as virgin animals treated with the carcinogen. (Am J Pathol 1980, 100:497-512)

ALTHOUGH the cause of breast cancer is not known,[1] epidemiologic observations indicate that there are factors that exert either a protective or a stimulating influence on the development of breast cancer in women.[1-4] Among the protective factors are a first full-term pregnancy before 24 years of age and a late menarche.[1,5-8] Among the risk factors are nulliparity,[9-12] late pregnancy,[1] early menarche,[6,13] and abortion.[1,5,14-16]

The mechanism(s) by which a first full-term pregnancy confers protection and the influence of nulliparity and pregnancy interruption on the

From the Experimental Pathology Laboratory, Department of Biology, Michigan Cancer Foundation, Detroit, Michigan.

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Address reprint requests to Jose Russo, MD, Experimental Pathology Laboratory, Michigan Cancer Foundation, 110 E. Warren Avenue, Detroit, MI 48201.

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development of breast cancer later in life are not known.[1-16] The use of an experimental system that mimics the human situation, such as the induction of mammary gland carcinomas by administration of 7,12-dimethylbenz(a)anthracene (DMBA) to young virgin rats, has provided some insights into the role of reproduction in the susceptibility to carcinogenesis.[17,18] Nulliparous rats are highly susceptible to induction of mammary carcinomas by DMBA due to the presence in the mammary gland of undifferentiated structures called terminal end buds (TEBs), which are the site of origin of rat mammary carcinomas.[19-21] The susceptibility of TEBs to neoplastic transformation has been attributed to the fact that they are composed of an epithelial population with a large proliferative compartment, the cells of which cycle every 10 hours [22] and actively bind DMBA to DNA.[23] During pregnancy, the TEBs differentiate to alveolar buds (ABs) and lobules,[17] resulting in a progressive disappearance of undifferentiated structures, which are totally eliminated during lactation. As a consequence, the proliferating compartment of the gland is markedly reduced, and those cells which remain in cycle have a markedly lengthened G_1. These cells are able to repair DMBA-damaged DNA [24] and have a lower affinity for binding DMBA to DNA.[23]

These structural and physiologic changes induced in the mammary gland by pregnancy and lactation result in a lower susceptibility to carcinogenesis, which is reflected in the almost total refractoriness of parous rat mammary gland to the development of carcinomas after DMBA administration.[19,21]

In the present work we have used the experimental system described above to study the influence of pregnancy interruption on the development of the mammary gland and consequently on its susceptibility to carcinogenesis induced by DMBA administration.

Materials and Methods

Animals

Noninbred, mature male and virgin female Sprague–Dawley rats were obtained from Spartan Research Animals, Inc., Haslett, Michigan. They were housed 4 to a cage and received water and food *ad libitum*. The environment was controlled for temperature at 24 ± 1 C and for 12 hours of light and 12 hours of darkness. The rats were separated into 5 groups. Groups I, II, and III consisted of 11 females each. At 50 days of age they were mated with 90-day-old males; they were housed 2 females and 1 male to a cage. After mating was confirmed by the presence of either a vaginal plug or sperm in the vaginal smear, the males were removed from the cages. In Group I all the females were allowed to complete pregnancy and to nurse their litters for 21 days; after weaning, the mothers were maintained in isolation for 21 days to allow postlactational involution of the mammary gland. In Group II the females completed pregnancy, but after delivery they were not permitted to nurse their offspring. The mothers were maintained in isolation for 21 days to

allow postpregnancy involution of the mammary gland. The pregnant females of Group III were subjected to hysterectomy, with removal of all the fetuses on the 12th day of gestation. We visually verified that in this group, both ovaries, with intact circulation, were left in place in each animal. The animals were maintained in isolation for 21 days to allow mammary gland involution. Group IV consisted of 17 virgin females age-matched with Group I (120 days old), and Group V consisted of 9 virgin females age-matched with Groups II and III (80–90 days of age).

DNA Labeling Index

Two animals from each group received, while in estrus, an intraperitoneal injection of 2.5 μCi methyl-^{3}H-thymidine/g body weight (methyl-^{3}H-thymidine-specific activity 25 Ci/mM, Amersham Searle, Arlington Heights, Ill). The animals were killed 1 hour after injection. The mammary glands were removed and processed for autoradiography, and whole mount as described previously.[19,20]

Carcinogen Treatment

The remaining animals of each group received, while in estrus, a single intragastric dose of 10 mg DMBA/100 g body weight (7,12-dimethylbenz[α]anthracene, Eastman Organic Chemicals, Rochester, NY). All inoculated animals were palpated weekly and killed 22 weeks after DMBA administration.The mammary glands and tumors were dissected from the skin and processed for whole mount and light microscopy as described elsewhere.[19,20]

Microscopic Analysis

The DNA labeling index (DNA-LI) was determined by counting of the number of labeled nuclei and the total number of cells that composed the epithelium of those terminal end buds (TEBs), terminal ducts (TDs), and alveolar buds (ABs) that could be clearly identified in either longitudinal or cross-sections. The identification of these structures was based upon the application of criteria previously described.[19,20] An average of 6 slides per gland per animal was counted. The DNA-LI was expressed as the number of labeled nuclei per 100 epithelial cells. The values obtained for each of the mammary glands were pooled and expressed as an average value for each animal.

Tumor Quantification

The criteria used have been described previously.[19]

Results

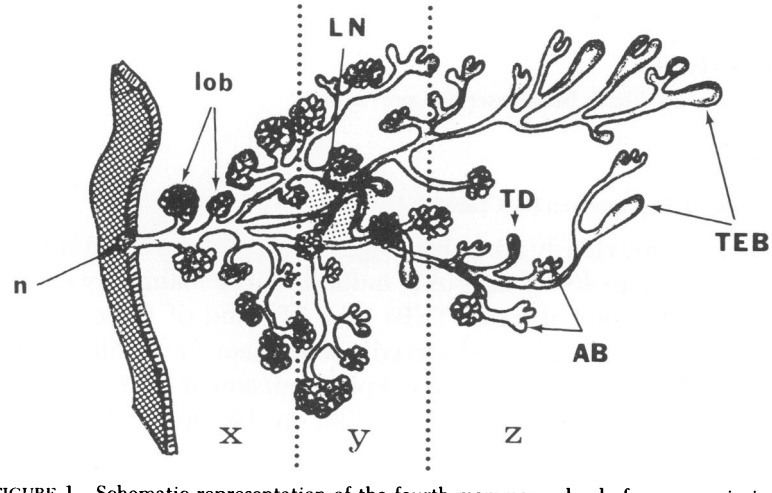
Mammary Gland Structure and DNA Labeling Index

As we have previously described,[17] pregnancy occurring at a young age (ie, when rats were 45–55 days old), induced in the mammary gland to undergo rapid differentiation of TEBs into ABs and of these into lobules. This phenomenon was not observed simultaneously in all areas of the gland, but instead occurred as a focal phenomenon, in which the degree of differentiation achieved varied depending on the topographic area of the gland examined. For this reason we divided the mammary gland into three arbitrarily chosen topographic areas: X, the area round the nipple; Y, the middle portion of the gland adjacent to area X and that contained the inguinal lymph nodes when the fourth mammary gland was consid-

ered; and Z, the most distal portion of the gland, containing the terminal branches of the mammary gland tree (Text-figure 1).

The most profuse branching and sprouting of ABs and lobules after pregnancy occurred in Area X (Figure 7), followed by area Y. The mammary glands of animals undergoing one pregnancy and lactation (Group I) and those of animals completing a full-term pregnancy (Group II) presented extensive alveolar development also in area Z ((Figures 1, 2, 3, and 4); no TEBs were observed in any area of the gland, and only occassional TDs were present in area Z. The DNA-LI in TDs was not significantly different in these two groups of animals, but it was significantly lower than that of control animals (Groups IV and V). In ABs and alveoli, the DNA-LI was also similar in both groups of animals, and these values were not significantly different from those of control animals (Groups IV and V) (Table 1).

By the 12th day of pregnancy there were still present in area Z numerous TEBs and ABs that never reached complete differentiation due to the interruption of pregnancy (Figures 5 and 8). After the period of involution, these topographic differences were still observed. Areas X and Y contained numerous small lobules and moderate numbers of ABs, but not TEBs (Figures 6 and 7). In section Z, ABs and lobules were also present, but in a lower number than in areas X and Y, and numerous TEBs were observed; these TEBs were morphologically identical to those of young virgin and age-matched control rats. Although DNA-LI was lower in



TEXT-FIGURE 1—Schematic representation of the fourth mammary gland of a young virgin rat arbitrarily divided in three different topographic areas: *X*, *Y*, and *Z*. Nipple, *n*; lymph node, *LN*; lobules, *lob*; alveolar buds, *AB*; terminal end buds, *TEB*; terminal ducts, *TD*.

Table 1—DNA Labeling Index of the Different Histologic Compartments of the Rat Mammary Gland at the Time of DMBA Administration

Group	Age*	Terminal end buds		Terminal ducts		Alveolar buds	
		Cells counted	DNA-LI (± SD)†	Cells counted	DNA-LI (± SD)†	Cells counted	DNA-LI (± SD)†
I (P + L)	120	—	—	2308	0.28 ± 0.10	38,970	0.11 ± 0.03
II (P)	92	—	—	9986	0.33 ± 0.15	46,798	0.19 ± 0.11
III (P int)	83	1898	11.4 ± 3.20	5104	0.45 ± 0.31	24,620	0.08 ± 0.02
IV (Vi)	120–140	2838	16.6 ± 6.05	5492	2.60 ± 0.90	8420	0.13 ± 0.10
V (Vi)	80–90	2146	16.7 ± 9.30	4732	11.95 ± 4.90	6545	0.30 ± 0.21

P + L = pregnancy + lactation, followed by a resting period of 21 days; P = pregnancy alone, without lactation, followed by a resting period of 21 days, P int = pregnancy interrupted at the 12th day, followed by a resting period of 21 days, Vi = control age-matched virgin rats.

* Age in days at which the rats received the DMBA inoculation.

† Values are mean ± SD. The Students *t* test was used for all possible comparisons. For DNA-LI, the following comparisons were significantly different ($P < 0.001$): terminal ducts of Groups I, II, and III *vs* IV and V; Group IV *vs* V.

TEBs of these experimental animals (Group III) than in the same structures of control animals (Group V), the difference was not statistically significant (Table 1). On the other hand, the DNA-LI of TDs of animals in which pregnancy was interrupted was similar to the DNA-LI of TDs and ducts of animals completing one pregnancy (Group II) or pregnancy and lactation (Group I). ABs and alveoli in area X, close to the nipple, were as well developed as those present in the mammary glands of animals that had completed one pregnancy or pregnancy and lactation (Groups I and II). (Compare Figure 7 with Figures 1 and 2.) Besides appearing equally developed, ABs and alveoli of Group III animals had a DNA-LI similar to that of control and experimental groups (Table 1).

Tumorigenic Response

In control virgin rats receiving the carcinogen at the age of 120–140 days (Group IV) and 80–90 days (Group V) a total of 25 and 13 carcinomas developed, respectively, with an incidence of 66.7% for Group IV and 71.4% for Group V (Table 2). None of the animals that underwent pregnancy alone developed mammary gland carcinomas. In the group of animals that completed one pregnancy and lactation only 1 animal developed a carcinoma (Table 2). Seventy-seven percent of those animals in which pregnancy was interrupted developed mammary gland carcinomas

Table 2—Incidence of Adenocarcinomas in DMBA-Treated Sprague–Dawley Rats

Group	Age*	Number animals with tumor/total number of animals	% Animals with tumor	Total number of tumors	Number of tumors per tumor-bearing animal
I (P + L)	120	1/9	11.1	1	1.0
II (P)	92	0/9	0.0	0	0.0
III (P int)	83	7/9	77.7	15	2.1
IV (Vi)	120–140	10/15	66.7	25	2.5
V (Vi)	80–90	5/7	71.4	13	2.6

P + L = pregnancy + lactation, followed by resting period of 21 days, P = pregnancy alone, without lactation, followed by a resting period of 21 days, P int = pregnancy interrupted at the 12th day, followed by a resting period of 21 days; Vi = control age-matched virgin rats.

* Age in days at which the rats received the DMBA inoculation.

and with essentially the same number of tumors per animal as the age-matched control rats (Group V).

In all animals inoculated with DMBA benign lesions developed (Table 3). Pregnancy and lactation, even after a resting period, considerably decreased the number of benign lesions when the rats were compared with control age-matched rats (Group IV). Those animals that underwent one pregnancy alone without lactation had more fibroadenomas, hyperplastic alveolar nodules (HANs), and cysts per animal and per gland than the control group (V) and considerably more than animals with one pregnancy plus one lactation (Table 3). In those animals in which pregnancy was interrupted (Group III), the number of benign lesions (adenomas, fibroadenomas, and HANs) was slightly higher than in age-matched controls, threefold higher than in animals with pregnancy and lactation, but lower than in 120-day-old virgin (Group IV) animals and animals with one pregnancy without lactation (Group II) (Table 3).

Discussion

Our results show that a full-term pregnancy itself renders the mammary gland less susceptible to DMBA carcinogenesis, and that, although lactation is not required for complete protection, as has been suggested by other authors,[25-27] animals that have not lactated are more prone to develop benign tumors. Although several authors have observed that pregnancy acts as a protective factor in carcinogenesis,[18,28,29] the mechanism of this protection was not clearly understood until a more precise knowledge of the pathogenesis of the disease was obtained.[17,20] Pregnancy stimulates differentiation of the TEBs and TDs of the virgin rat mammary gland to ABs and lobules, thus eliminating or reducing the number of target sites for carcinogenesis.[17,19]

We show here that, in order to be protective, the development of the gland must be complete. Pregnant [30] or lactating rats [28] treated with chemical carcinogens respond with a significant reduction in mammary tumor incidence, while pregnancy interruption gives no protection at all. This is due to the fact that in the mammary gland of animals in which pregnancy has been interrupted, the glands contain some areas with completely differentiated structures and others in which undifferentiated structures prevailed.

It has been observed that the hormonal changes of pregnancy accelerated tumor developement in Sprague–Dawley rats mated 15 days after the administration of DMBA.[18] In contrast, a single pregnancy prior to feeding the carcinogen, as well as pregnancy followed by nursing, reduced the percentage of incidence and increased the latency period of tumor de-

Table 3—Incidence of Microscopic Benign Lesions in DMBA-Treated Rats

Group	Age*	Number of animals	Number of glands studied	Intro-ductal papil-lomas	Ade-nomas	Fibro Ade-nomas	Hyperplastic Alveolar nodules (HA)	Cysts	Total number of lesions	Number of lesions per gland
I (P + L)	120	9	32	5	0	1	28	10	39	1.2
II (P)	92	9	37	0	1	10	185	21	217	5.9
III (P int)	83	9	36	3	5	8	102	4	119	3.3
IV (Vi)	120–140	15	61	0	3	16	216	32	267	4.3
V (Vi)	80–90	7	29	0	0	1	46	35	82	2.8

P + L = pregnancy + lactation, followed by a resting period of 21 days; P = pregnancy alone, without lactation, followed by a resting period of 21 days; P int = pregnancy interrupted at the 12th day, followed by a resting period of 21 days; Vi = control age-matched virgin rats.

* Age in days at which the rats received the DMBA inoculation.

velopment.[17,28,29] Transplants of pituitary homografts, progesterone,[31] or combinations of progesterone and estrogen administered to intact rats for 20 days before DMBA [32] partially inhibited tumor development; this finding is explained by the fact that these treatments resulted in enhanced growth of the mammary gland by the time of carcinogen treatment. However, the protective effect is much lower than that induced by full-term pregnancy, indicating that other hormones besides estrogen and progesterone must act in this process.

The placenta participates in rat mammary gland growth during pregnancy.[33] Exogenous administration of human placental lactogen to adreno-ovariectomized animals promotes normal mammary glandular growth comparable to that of intact animals.[34] Under normal conditions, placental lactogen has a direct effect on the mammary gland, as well as an indirect one, by promoting the secretion of progesterone through its luteotropic effect on ovaries. Equine gonadotropin, administered prior to carcinogen treatment, produces a gestational or lactational type of mammary hyperplasia that considerably reduces the incidence of tumors but does not cause a complete inhibition.[30,35] Although the stimulated mammary gland in the rat is relatively refractory to carcinogen treatment, the hormones of pregnancy and lactation do not have to be present, since mammary glands that have regressed following pregnancy or nursing, as occurs in our experimental animals, are still refractory to carcinogenesis. This finding indicates that it is not only the hormonal status of the rat that is responsible for this refractoriness, but permanent structural changes that are mediated by the hormones of pregnancy and lactation and that remain after the hormones have returned to nonpregnant physiologic levels. These permanent changes consist of the replacement of TEBs by ABs and alveoli, which result in a decrease in the proliferative compartment of the gland.[22,24] When the stimulus of pregnancy is interrupted, the differentiation of the gland is not completed, as is also true when different hormonal treatments that simulate pregnancy are given.[30,35-37]

In women, pregnancy is protective when it occurs before age 24.[1] In contrast, abortion is associated with increased risk of carcinomas of the breast.[5,14-16] The explanation for these epidemiologic findings is not known, but the parallelism between the DMBA-induced rat mammary carcinoma model and the human situation is striking. It has been shown that in women during gestation, prolactin from the pituitary [38] and placenta,[33] as well as the secretion of estrogen and progesterone,[39-41] increase, and they act synergistically to promote breast growth and differentiation. Abortion would interrupt this process, leaving in the gland

undifferentiated structures like those observed in the rat mammary gland, which could render the gland again susceptible to carcinogenesis.

The number of benign lesions in the mammary gland increases with increasing age at the time of DMBA administration.[19] Pregnancy alone without lactation as well as pregnancy interruption, both followed by a resting period of 21 days prior to carcinogen administration, result in a similar or even higher incidence of benign lesions when the rats are compared with control virgin rats of matching age. Therefore, while lactation seems to have no additional effect on protecting the mammary gland from DMBA carcinogenesis, it seems to have a favorable effect for preventing the formation of HANs and cysts. In women, lactation per se does not seem to protect the breast from the development of cancer, but its effect on the incidence of benign lesions is not known.[42-44] The mechanism by which pregnancy and lactation reduce the susceptibility of the rat to benign lesions has been explained as the consequence of a diminution in the size of the proliferative compartment and a lengthening of the G_1 phase of the cell cycle in epithelial cells of ABs,[24] the site of origin of these lesions.[20] Even though pregnancy alone and pregnancy interruption decreased the DNA-LI of the AB's epithelial cells, the extent of the changes at the level of both growth fraction and cell cycle is not known. Other factors, such as metabolic activation of carcinogens, DMBA–DNA binding, and DNA repair, that might be modified by the reproductive experience of the host, are now under study.

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[*Illustrations follow*]

Figure 1—Histologic section of the mammary gland of Group I rat showing extensive alveolar development. (H&E, ×25)

Figure 2—Histologic section of the mammary gland of a Group II rat. The alveolar development is similar to that of Group I (Figure 1). (H&E, ×25)

Figure 3—Whole mount of the mammary gland of a Group I rat after 21 days of postlactational involution. (Toluidine blue, ×25)

Figure 4—Histologic section of the lobular structures shown in Figure 3. (H&E, ×80)

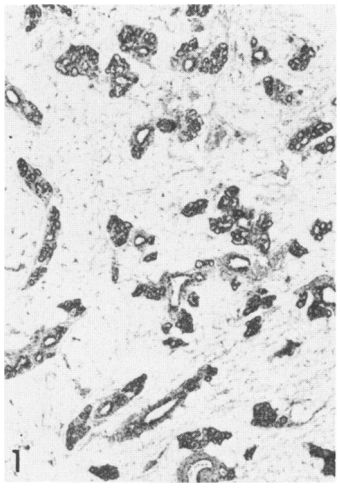
Figure 5—Whole mount of the mammary gland of a Group III rat. Distal portion (*Z*) of the gland with TEBs, TD, and scarce ABs. (Toluidine blue, ×25)

Figure 6—Histologic section through area *Y* of the mammary gland of a Group III rat. (H&E, ×25)

Figure 7—Histologic section through area *X* of the mammary gland of a Group III rat. (H&E, ×25)

Figure 8—Histologic section through area *Z* of the mammary gland of a Group III rat. (H&E, ×25)

Figures 9 and 10—Autoradiograph of a cross-section of a TEB located in area *Z* of the mammary gland of a Group III rat. (H&E, ×80)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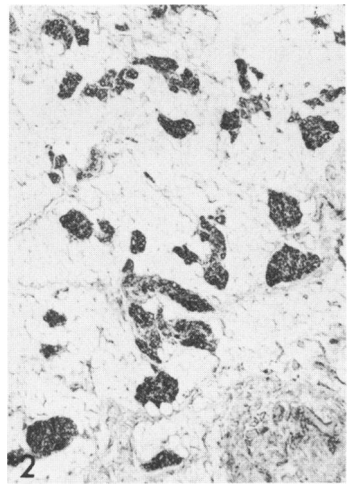
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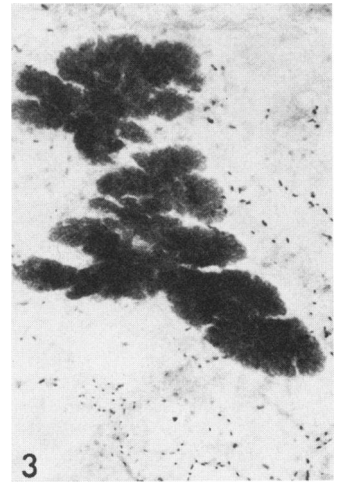
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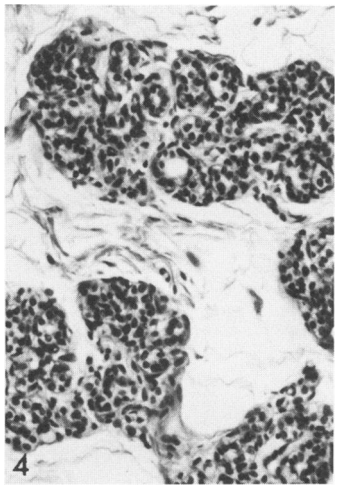
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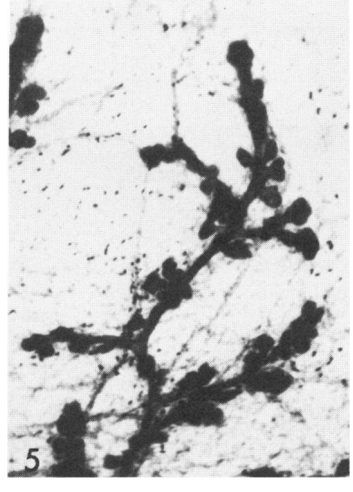
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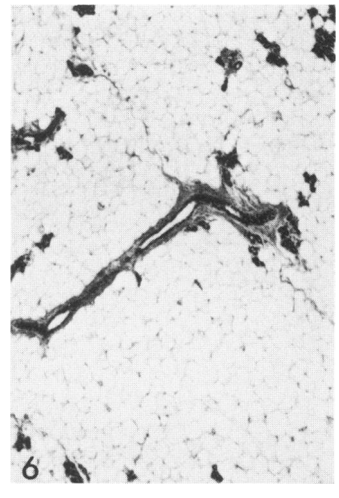
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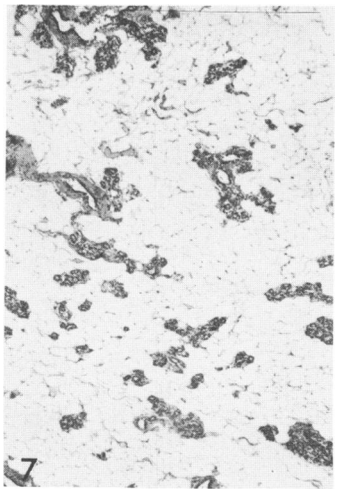
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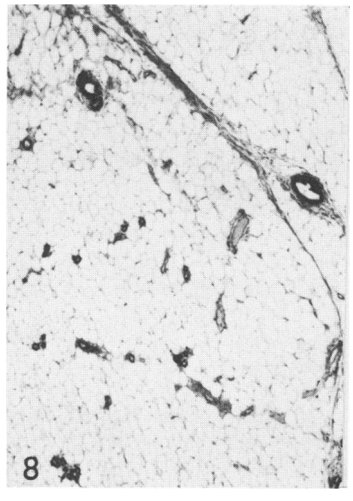
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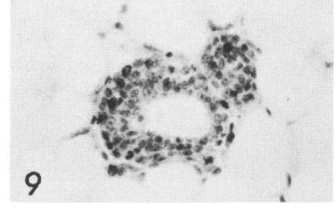
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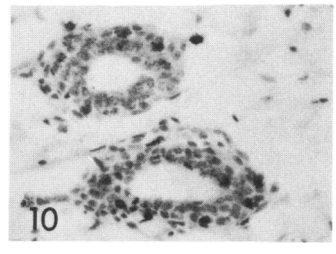
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